

In vivo ^{13}C NMR assessment of brain glycogen concentration and turnover in the awake rat

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Abstract

Brain glycogen metabolism was recently observed in vivo and found to be very slow in the lightly α -chloralose anesthetized rat [J. Neurochem. 73 (1999) 1300]. Based on that slow turnover, the total glycogen content in the awake rat brain and its turnover time were assessed after administering ^{13}C -labeled glucose for 48 h. Label incorporation into glycogen, glucose, amino acid, and *N*-acetyl-aspartate (NAA) resonances was observed. The amount of ^{13}C label incorporated into glycogen was variable and did not correlate with that in glutamate ($r = -0.1$, $P > 0.86$). However, the amount of ^{13}C label incorporated into glycogen was very similar to that in NAA ($r = 0.93$), implying similar turnover times between brain glycogen and NAA (~ 10 h). Absolute quantification of the total concentration of brain glycogen in the awake, normoglycemic rat yielded $3.3 \pm 0.8 \mu\text{mol/g}$ ($n = 6$, mean \pm S.D.).

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1. Introduction

Glucose is the main substrate for brain energy metabolism. However, an endogenous store of carbohydrates, brain glycogen, can serve as an energy reserve when glucose supply is limited. The concentrations of brain glycogen can be influenced by glucose (Goldberg and O'Toole, 1969; Nelson et al., 1968), anesthetics (Nelson et al., 1968; Nordstrom and Siesjo, 1978) and neurotransmitters (Magistretti et al., 1986; Pellerin and Magistretti, 1994). It has also been suggested that brain glycogen metabolism may be affected by neuronal activity such as focal physiological stimulation (Swanson, 1992).

To date, brain glycogen concentrations were mainly measured by invasive methods such as biochemical analysis and enzymatic analysis. Due to rapid postmortem degradation of brain glycogen, quantitative determination of glycogen content in the brain is rather challenging (Cruz and Dienel, 2002; Kong et al., 2002). However, brain glycogen can be measured using in vivo ^{13}C nuclear magnetic resonance

(NMR) in conjunction with infusion of ^{13}C -labeled glucose. We recently demonstrated (Choi et al., 1999) that serial measurements of brain glycogen in vivo over 16 h are possible from a single animal using specifically designed three-dimensional localized ^{13}C NMR methods to ensure that extra-cerebral glycogen does not contaminate the signal (Choi et al., 2000). In our previous study, we observed a maximal concentration of $[1\text{-}^{13}\text{C}]$ brain glycogen of $5.1 \mu\text{mol/g}$ during hyperglycemia and that the steady-state turnover rate was very slow, $0.5 \mu\text{mol}/(\text{g h})$, in the lightly α -chloralose anesthetized rat brain in vivo (Choi et al., 1999).

Considering that anesthetics and brain glucose content have been reported to affect glycogen metabolism, we became interested in assessing total brain glycogen content in the awake normoglycemic brain. Therefore, we sought to estimate (a) the total concentration of brain glycogen and (b) the metabolic rate of brain glycogen in the awake normoglycemic animal using ^{13}C NMR methods. To achieve these aims, we employed a novel strategy, namely the comparison of the amount of ^{13}C label incorporated into glycogen with that into amino acids, i.e. glutamate and NAA, while administering ^{13}C -labeled glucose for 2 days. A preliminary account of part of this work has appeared (Choi and Gruetter, 2001).

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2. Materials and methods

2.1. Animal preparation

The study was approved by the Institutional Animal Care and Use Committee (IACUC) and conducted according to the guidelines for the care and use of laboratory animals at the University of Minnesota. Male Sprague-Dawley rats (241 ± 11 g, mean \pm S.E., $n = 6$) were fasted overnight with free access to water prior to the study to minimize the amount of unlabeled glycogen in the liver. The animals were then allowed access to a 99%-enriched [$1\text{-}^{13}\text{C}$] glucose solution (5–10%, w/v) ad libitum, which was the only exogenous carbon source for at least 48 h. After 2 days, animals were anesthetized using 2% isoflurane (Marsam Pharmaceuticals, Cherry Hill, NJ) in a 1:1 mixture of nitrous-oxide (N_2O) and oxygen (O_2) gases for surgery to insert a catheter into the left femoral vein for a bolus intravenous infusion of α -chloralose (24 mg/kg body weight). After preparation, which lasted less than 1 h, animals were immediately placed in the magnet and the ^{13}C NMR measurements begun.

2.2. NMR methods

All experiments were performed on a 9.4 T, 31-cm horizontal-bore magnet (Magnex Scientific, UK), interfaced to a Varian INOVA console (Palo Alto, CA, USA). An actively shielded gradient coil (Magnex Scientific, Abingdon, UK) with an 11-cm inner diameter was used and a home-built quadrature ^1H surface RF coil (14-mm diameter) and a linearly polarized three-turn ^{13}C RF coil (12-mm diameter) were used as a transceiver for ^1H NMR and ^{13}C NMR as described previously (Adriany and Gruetter, 1997). A 99% ^{13}C -enriched formic acid sphere was located at the center of the linearly polarized coil as an external reference and the coil was placed on the animal's head (Choi et al., 2000). Automated localized shimming with a fully adiabatic version of FAST(EST)MAP was used (Gruetter and Tkac, 2000). The full-width-at-half-maximum of the in vivo water signal was about 20 Hz in a nominal 510- μl volume. The volume of interest (VOI) was placed in the brain and a 510- μl volume was the typical size of the nominal VOI. Three-dimensional localization based on outer volume saturation using the SIRENE method (Choi et al., 2000) combined with adiabatic excitation and WALTZ-16 ^1H decoupling during the 100-ms acquisition time was used as in previous studies (Choi et al., 1999, 2000). The efficiency of localization was verified from the absence of the natural abundance lipid signal (30.5 ppm) in spectra optimized for the amino acid region (20–40 ppm) as the lipid signals are not detectable in the normal brain in vivo. The localized signals of glycogen and glucose were acquired over 25 min and the spectrometer offset was set to 100 ppm (repetition time, TR = 1 s, 64 scans). The acquisition of localized signals from the amino acid region was interleaved with spectrometer offset set to 30.5 ppm (TR =

1.5 s, 64 scans) to minimize chemical shift displacement errors.

2.3. Comparison of turnover rates between glycogen and amino acids

The standard approach to measure or estimate a metabolic rate is to measure the temporal changes in the precursor isotopic enrichment, such as plasma glucose, and to use this information as input to the modeling of the measured rate of labeling of the product P. For the rat studies performed over 48 h in this study, this was not a feasible approach. Instead, we propose to estimate the rates of metabolism relative to those from another compound with a known turnover time, based on the following argument: first, the rate of label incorporation from a precursor (e.g. plasma glucose or acetyl-CoA) to a metabolic product P is given according to tracer methodology (e.g. Eq. (1) in Gruetter et al., 1998a,b). For the present calculation, a metabolic steady state was assumed, i.e. it was assumed that the rate of label incorporation is dominated by turnover (label cycling in and out of the metabolite) rather than net concentration changes, as is the case for the brain amino acids. Brain glycogen metabolism may also be at a quasi-steady state, because it is unlikely that brain glycogen concentration accumulates steadily over the two full day/night cycles measured here. At metabolic steady state, concentration changes are small and a rapid rise in isotopic enrichment of the precursor from natural abundance (0.011) to a new value IE_0 (assuming $\text{IE}_0 \gg 0.011$) results in a slower labeling of the product with a time course for the isotopic enrichment for metabolite, $\text{IE}_\text{P} = {}^{13}\text{P}(t)/\text{P}$, that is given by

$${}^{13}\text{IE}_\text{P}(t) = \text{IE}_0(1 - e^{-t/\tau}) \quad (1)$$

where the time constant τ is the turnover time which is related to the label half-life $\tau_{1/2} = 0.7\tau$ and is given by $\tau = \text{P}/V$. P is the pool size (in $\mu\text{mol/g}$) and V the turnover rate or metabolic rate (in $\mu\text{mol/g min}$). The amount of label incorporated into two distinct metabolites with similar turnover times will thus change very similarly in time.

Label incorporation into glucose, glutamate and NAA occurs with distinctively different time scales with half-life $\tau_{1/2}$ on the order of ~ 10 min, 1–2 h and ~ 14 h, respectively (Choi and Gruetter, 2000; Hyder et al., 1996; Moreno et al., 2001; Pfeuffer et al., 1999a), according to the relative metabolic rates and pool sizes. It has been reported that the turnover of NAA is very slow (Gruetter et al., 1998a,b; Kunnecke et al., 1993) with some studies reporting rates on the order of 0.5 $\mu\text{mol/(g h)}$ based on ^{13}C NMR studies with ^{13}C -labeled glucose infusion (Choi and Gruetter, 2000; Moreno et al., 2001).

2.4. Quantification of NMR signals

The ^{13}C NMR signals of brain glycogen (in μmol glucosyl units/g wet weight), glucose, glutamate and NAA

were quantified using the external reference method (Choi et al., 1999; Gruetter et al., 1998a,b). In brief, in vivo ^{13}C NMR signals of glycogen, glucose, glutamate and NAA were quantified by comparison with measurements of phantom solutions (pH = 7.2) containing ~400 mM glucosyl units of natural abundance oyster glycogen, 0.9 mM of 99%-enriched $[1-^{13}\text{C}]$ D-glucose, 250 mM of glutamate and 250 mM of NAA after correction for the effects of loading and nuclear Overhauser effect on the NMR signals.

To measure the isotopic enrichment of NAA, the total NAA concentration, $[\text{NAA}]$, was set to $8.5 \mu\text{mol/g}$ based on quantification of $[\text{NAA}]$ in ^1H NMR spectra obtained from three separate animals using LCModel (Pfeuffer et al., 1999b) yielding $8.5 \pm 0.1 \mu\text{mol/g}$ (mean \pm S.E.) in excellent agreement with previous studies (Pfeuffer et al., 1999b). ^1H NMR spectra were acquired with a signal-to-noise ratio of at least 30:1 in 8 min from a $150 \mu\text{l}$ volume in the brain using a STEAM sequence with 2-ms echo time, 5-s repetition time, and 20-ms mixing time (Tkac et al., 1999).

The concentration of total brain glycogen was calculated as follows: based on the excellent correlation of the label incorporated into glycogen, $^{13}\text{Glyc}_1$, and into NAA C6, $^{13}\text{NAA}_6$, the isotopic enrichment of glycogen, $\text{IE}_{\text{Glyc}} = ^{13}\text{Glyc}_1/[\text{Glyc}]$, was assumed to be proportional to that of NAA, $\text{IE}_{\text{NAA}} = ^{13}\text{NAA}_6/[\text{NAA}]$. IE_{NAA} was calculated from the quantified ^{13}C NMR signal of $^{13}\text{NAA}_6$ C6, $^{13}\text{NAA}_6$, and the average total NAA concentration, $[\text{NAA}]$, which is known to be stable under normoxic conditions. Because the breakdown of the hexoses into two trioses in glycolysis results in pyruvate C3 labeling from the C1 as well as the C6 of glucose, the isotopic enrichment of acetyl-CoA (precursor of $^{13}\text{NAA}_6$) can be at most half of that of glucose (precursor of Glyc_1). Therefore, we assumed that the isotopic enrichment of NAA was half of that of glycogen, and total brain glycogen was calculated from Eq. (2):

$$[\text{Glyc}] = \frac{^{13}\text{Glyc}_1}{2\text{IE}_{\text{NAA}}} \quad (2)$$

where $[\text{Glyc}]$ denotes absolute concentration of glycogen in the brain and $^{13}\text{Glyc}_1$ denotes the concentration of ^{13}C -labeled glycogen C1 ($\mu\text{mol/g}$ glucosyl units). This quantification assumed that based on the stable $[\text{NAA}]$ and the slow metabolism of NAA, $0.6 \mu\text{mol/(g h)}$ (Choi and Gruetter, 2000; Moreno et al., 2001), the change of

^{13}C -labeled NAA concentration and thus IE_{NAA} was negligible during the time of acquisition (<30 min).

3. Results

Following 2 days of ad libitum administration of ^{13}C -labeled glucose (Fig. 1), the ^{13}C NMR spectra of the brain showed in all animals clearly visible labeling of glycogen, NAA, glutamate (Glu), glutamine (Gln) and aspartate (Asp) and, in most cases, glucose (Glc). The degree of labeling was, however, variable, which implied that the isotopic enrichment of the precursor glucose varied between animals and possibly in time as well.

The amount of ^{13}C label incorporated into brain glycogen is most likely to correlate with the amount of label incorporated into that amino acid, which has a label half-life similar to that of glycogen. Therefore, these widely different turnover times of amino acids can serve as a reference to estimate the turnover rate of brain glycogen in the awake rat brain by comparing the amount of ^{13}C label in brain glycogen with that in glutamate and NAA, respectively.

The correlation between the amount of label incorporated into brain glycogen C1 and the amount of label incorporated into glutamate C4 following ad libitum administration of ^{13}C -labeled glucose was not statistically significant ($r = -0.1$, $P > 0.86$ for correlation, dotted line in Fig. 2A), indicating that glutamate turnover was faster than brain glycogen turnover. In contrast, an excellent correlation between the concentrations of ^{13}C -labeled brain glycogen C1 and ^{13}C -labeled NAA C6 was observed with $r = 0.93$, $P < 0.01$ for correlation (solid line in Fig. 2B). Therefore, from the observation of a slow rate of ^{13}C label incorporation into NAA similar to that of brain glycogen (Choi and Gruetter, 2000), it was assumed that the isotopic enrichment of NAA was approximately half that of brain glycogen after more than 2 days of $[1-^{13}\text{C}]$ glucose administration.

Total concentration of brain glycogen (closed squares in Fig. 2C) was calculated to be $3.3 \pm 0.8 \mu\text{mol/g}$ (mean \pm S.D., $n = 6$) using Eq. (2) based on the estimated isotopic enrichment of NAA and the stable concentration of total NAA. The glycogen concentration on the order of $\sim 3 \mu\text{mol/g}$ falls well into the range of published concentration values (Table 1).

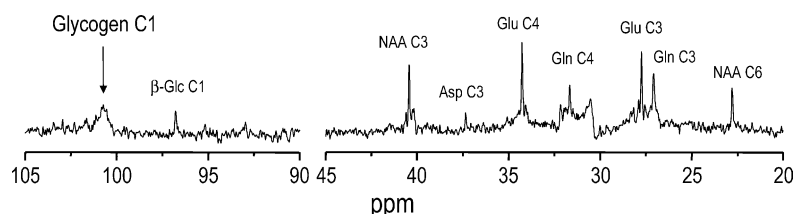


Fig. 1. Label incorporation into brain glycogen and amino acids following administration of ^{13}C -labeled glucose for 2 days. Label incorporation into glycogen and glucose C1, as well as several metabolites (*N*-acetyl-aspartate (NAA), aspartate (Asp), glutamate (Glu), and glutamine (Gln)) was observed in a ^{13}C NMR spectrum acquired over 25 min from the rat brain after 99%-enriched $[1-^{13}\text{C}]$ glucose had been administered for over 48 h ad libitum. Processing consisted of 10 Hz line-broadening and zero-filling prior to Fourier transformation. The spectrum is shown without baseline correction.

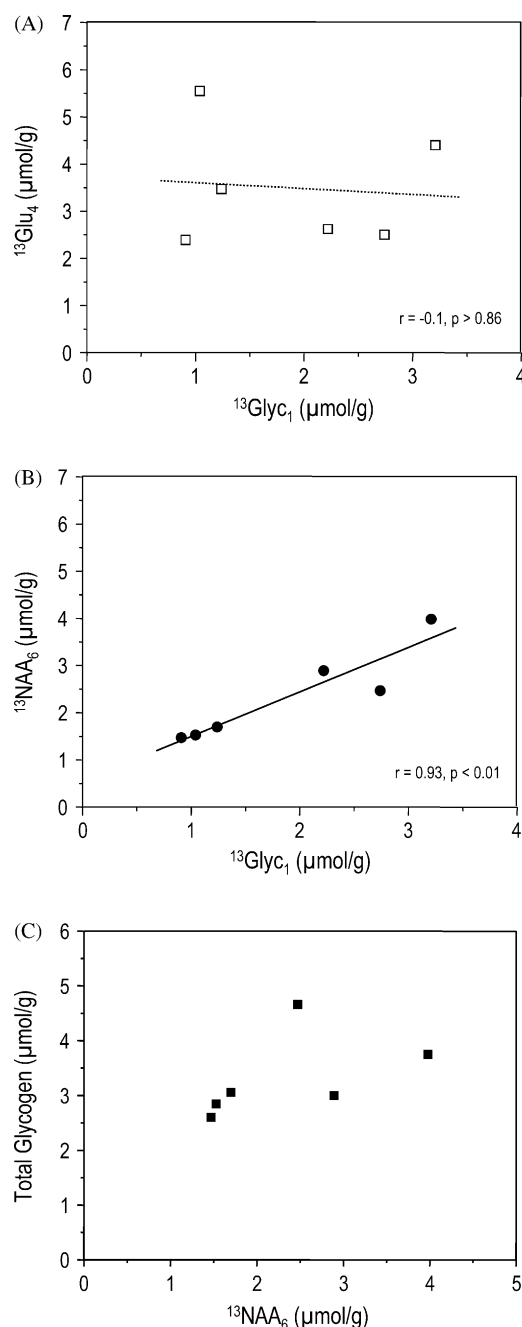


Fig. 2. Estimation of brain glycogen turnover times and total brain glycogen content. (A) Comparison of label incorporation into brain glycogen C1, $^{13}\text{Glyc}$, and glutamate C4 $^{13}\text{Glu}_4$ (□). The dotted line indicates the linear regression to the data ($r = -0.1$, $P > 0.86$, $n = 6$). (B) Comparison between label incorporation into brain glycogen C1 and NAA C6 (●). The solid line indicates the result of linear regression ($r = 0.93$, $P < 0.01$, $n = 6$). (C) Total glycogen concentration was calculated using Eq. (1) and found to be $3.3 \pm 0.8 \mu\text{mol/g}$ (mean \pm S.D., $n = 6$).

4. Discussion

This study demonstrates a novel experimental design, by measuring label incorporation into brain glycogen after administration of ^{13}C -labeled glucose for a period of days

Table 1

Brain glycogen concentration reported in rodents

Method	Glycogen ($\mu\text{mol/g}$)	Subject
^{13}C NMR	3.3 ± 0.8	Rat ^a
Microwave fixation	6 ^b	Rat ^c
Microwave fixation	~ 3 ^b	Rat ^d
Microwave fixation	3–4	Rat ^e
Anthrone method	4.2	Rat ^f
Liquid N_2	2.6–4.4 ^g	Rat ^h
Preserved brain tissue slice using nembutal anesthesia	3.1–4.7 ^g	Rat ^h
N/A	3.3	Rat ⁱ
Liquid N_2	1.7–2.8	Mouse ^j
Liquid N_2	~ 2.2	Mouse ^k
Liquid N_2	2.9	Mouse ^l

^a Present study.

^b Unit in the papers is given as nmol glucosyl units/mg protein. Unit conversion is based on protein content in the brain to be 11.7% of the brain wet weight.

^c Garriga and Cusso (1992).

^d Swanson (1992).

^e Kong et al. (2002).

^f Strang and Bachelard (1971).

^g Unit in the papers is given as mg/g wet wt, which is converted to μmol glucosyl units/g wet weight.

^h Taken from Table 2 in (Prasannan and Subrahmanyam, 1972 and references therein).

ⁱ Clarke and Sokoloff (1999).

^j Nelson et al. (1968).

^k Lowry et al. (1964).

^l Watanabe and Passonneau (1973).

(48 h) and comparing the amount of label incorporated into glycogen with that incorporated into other amino acids. From this, a new method, to non-invasively estimate total concentration of brain glycogen in vivo during chronic ingestion of ^{13}C -labeled substrates is proposed.

After 2 days of ^{13}C -labeled glucose administration ad libitum, labeling of glycogen, glucose and amino acids was observed in all animals. The amount of ^{13}C label incorporation into metabolites was variable, which can be readily explained by variability in the feeding cycle and variations in hepatic glucose production (gluconeogenesis) of each animal resulting in a varying isotopic enrichment of plasma glucose leading to a variable amount of ^{13}C label incorporation into glycogen, amino acids and NAA.

In principle, at isotopic steady state ($t \gg \tau$ in Eq. (1)), a variation of the isotopic enrichment IE_0 of the common precursor between animals will lead to a correlation in the relative amount of label incorporated into two metabolites, e.g. $^{13}\text{Glyc}_1$ and $^{13}\text{NAA}_6$ that merely reflects the variability in IE_0 between animals. However, in this case the amount of label in glutamate is expected to correlate also with that in glycogen, which was clearly not observed. Thus, it is highly likely that the variable $^{13}\text{NAA}_6$ reflects changes in the enrichment of the precursor that occurred in the time period prior to the NMR measurement. Therefore, the excellent correlation between the amount of label incorporated into brain glycogen and into NAA suggests that the turnover time of brain glycogen in the awake rat is comparable to

that of NAA, which is on the order of 10–14 h (Choi and Gruetter, 2000; Moreno et al., 2001) and much slower than that of glutamate, on the order of 1–2 h (Hyder et al., 1996; Pfeuffer et al., 1999a,b). From our data, we conclude that metabolism of brain glycogen in the awake rat brain must be very slow, which is in excellent agreement with our previous assessment of a basal brain glycogen turnover rate on the order of 0.5 $\mu\text{mol/g/h}$ in the α -chloralose anesthetized rat, with an estimated label turnover time of $\tau = 7\text{--}10$ h (Choi et al., 1999), which is comparable to that of NAA.

A study reported changes in the amount of radioactivity incorporated into glycogen during short periods of somatosensory stimulation in the awake rat (Swanson et al., 1992). It is important to recognize that our study assesses turnover on a much longer time scale of the total glycogen moiety and thus this turnover is conceivably smaller, as this has been shown for the limit dextrin (Watanabe and Passonneau, 1973). In addition, it is possible that only a part of glycogen molecules is activated during sensory stimulation that may constitute a fraction of the overall NMR signal of glycogen. In this context, it was interesting to note that in one case NAA C6 appeared to be labeled nearly close to 50% suggesting that the correspondingly high concentration of ^{13}C -labeled brain glycogen was nearly fully labeled. This is indicative of close to complete labeling of glycogen over 48 h. On the other hand, if the core of the glycogen molecule is not substantially labeled even over a long time period such as the 48 h in our study, the total brain glycogen concentration will be underestimated. In this case, because the outer-most tier of glycogen contains approximately 50% of all glucosyl units, the $\sim 3 \mu\text{mol/g}$ of brain glycogen measured in this study represent half of the total glycogen present. A putative concentration of 6 $\mu\text{mol/g}$ places our measurement into the upper range of concentrations reported (Table 1). A very low rate of turnover for the inner core of glycogen, however, fully supports the main conclusion of the paper, namely that turnover of the bulk brain glycogen pool is very slow in the awake rat. Considering the slow label turnover time, it is likely that major net glycogen concentration changes occur on an even slower time scale, which supports the role of brain glycogen in the proper recognition of hypoglycemia.

The amount of label incorporated into NAA was used to estimate the total brain glycogen concentration using Eq. (2). Our results indicate that the quantification of brain glycogen in vivo using ^{13}C NMR after prolonged administration of labeled glucose provides consistent values on the order of 3 $\mu\text{mol/g}$ in spite of a widely variable ^{13}C label incorporation into NAA C6, $^{13}\text{NAA}_6$ (Fig. 2C). Total brain glycogen was $3.3 \pm 0.8 \mu\text{mol/g}$, in excellent agreement with the literature values measured in conscious animals (Clarke and Sokoloff, 1999; Garriga and Cusso, 1992; Sagar et al., 1987; Strang and Bachelard, 1971) as shown in Table 1. This calculation was based on two assumptions, namely that the isotopic enrichment of the precursor for NAA C6, acetyl-CoA, was half that of the precursor of glycogen, glucose and that

brain glycogen metabolism was in a quasi-steady state. Dilution at the level of pyruvate or acetyl-CoA would lead to an underestimation of IE_{Glyc} and hence to an overestimation of the brain glycogen concentration. The upper limits of such a label dilution that occurs in such labeling studies can be estimated from glutamate, since the isotopic enrichment of acetyl-CoA is likely to be at least as high as that in glutamate. In the lightly anesthetized rat with metabolic rates closed to the awake condition, this dilution was reported at 25% (Hyder et al., 1996), which would lead to an underestimation of the glycogen concentration by 25%, but the quantification remains within the range of experimental values (Table 1). However, in the awake brain, where metabolism of glucose and glutamate turnover are much faster, this isotopic enrichment is expected to be higher, because of the relatively smaller contribution of potential diluting reactions. The strong labeling of NAA observed in one animal certainly supports the presence of a high isotopic enrichment in our study.

The second assumption in the quantification of brain glycogen concentration was that brain glycogen metabolism was assumed to be at a quasi-steady state. This was justified on the basis that over two day/night cycles, a persistent increase in brain glycogen metabolism is highly unlikely. Changes in brain glycogen content on the order of 30% appear possible following sleep deprivation (Kong et al., 2002). In our study, rats were fasted overnight prior to administration of the glucose, which has been shown not to affect brain glycogen concentrations substantially (Kong et al., 2002). However, if the feeding following fasting leads to net glycogen synthesis, such an increase in brain glycogen concentration would occur mostly at the beginning of the 48-h period of ^{13}C -labeled glucose administration. Such an early synthesis of glycogen thus can only influence the measurements if it persists for 2 days, which would further reinforce the main conclusion of this study, namely that brain glycogen metabolism is slow.

It is interesting to note that in our previous study after 4 h of $[1\text{-}^{13}\text{C}]$ glucose infusion, the amount of ^{13}C label incorporated into glycogen C1 was 5.1 $\mu\text{mol/g}$ (Choi et al., 1999), which is substantially more (35%) than the 3.3 $\mu\text{mol/g}$ of total brain glycogen measured in the present study ($P < 0.02$). It is likely that in the previous study, total brain glycogen was even higher as animals experienced modest hyperglycemia (13–16 mM plasma glucose), which likely resulted in hyperinsulinemia. Thus, the concentrations of glycogen C1 may reflect the combined effect of elevated plasma glucose and/or insulin to promote net glycogen synthesis in the brain, which is in agreement with studies in cell cultures (Nelson et al., 1968; Swanson et al., 1989). Therefore, comparison of the glycogen C1 in this study with that in our previous study supports our previous assertion that glucose infusion resulted in net glycogen synthesis (Choi et al., 1999).

Current sensitivities of MR experiments and the low brain glycogen content preclude natural abundance detection of

brain glycogen. Instead, ^{13}C -labeled glucose can be used to transfer label to the brain glycogen pool, which introduces turnover (substrate cycling in and out of glycogen) as a potential confounding factor. However, in the present study, potential confounding effects of label turnover in estimating total brain glycogen content were further reduced by referencing to a metabolite with a similar turnover time, namely NAA. Therefore, we conclude that brain glycogen metabolism in the normal conscious brain is as slow as NAA metabolism with complete turnover of the entire brain glycogen requiring more than a day.

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